Recent Res. Devel. in Agricultural & Food Chem., 1 (1997)

Solid phase extraction for the analysis of rodenticides in biological matrices

John J. Johnston, Thomas M. Primus and Dave A. Goldade
National Wildlife Research Center, U.S. Department of Agriculture, Analytical Chemistry, 3350 Eastbrook Drive, Fort Collins, CO 80525, USA

ABSTRACT

Solid phase extraction was utilized to develop selective cleanup methodology for the analysis of the indandione and coumarin anticoagulant rodenticides chlorophacinone and difethialone from complex biological matrices. A thorough understanding of matrix components and their potential interaction with solid phase supports and extraction solvent additives was essential for the development of these modern analytical methods. These methods are applicable to pesticide registration studies aimed at providing chemical agents to mitigate rodent damage to agriculture and forestry and to control the spread of rodent vectored diseases.

INTRODUCTION

Rodent Damage to U.S. Agriculture and the Need for New Rodenticides

Many rodents are considered pest species for a variety of reasons. Human population expansion frequently results in the conversion of wildlife habitat into monocultures with native plant species replaced by agricultural and/or urban environments(1). Rodents cause damage to agricultural crops such as sugar cane, sugar beets, vegetables, rice and fruit producing trees and vines. In a 1989 survey to assess the magnitude of wildlife induced losses to U.S. agriculture, 26% of the surveyed 20,000 farms and ranches reported losses related to rodent/rabbit activity (2). It is widely accepted that rodents play a significant role in postharvest food losses which wastes the entire investment required to plant, cultivate, protect, harvest and store the crop (3). Rodents such as pocket gophers (*Thomomys spp.*), ground squirrels (*Spermophilus spp.*) and prairie dogs (*Cynomys spp.*) frequently inhabit western U.S. rangelands and compete with livestock for vegetation (4).

Rodents also damage forest trees and impede reforestation efforts. In the southeastern U.S., beaver (*Castor canadensis*) is the primary wildlife species causing timber damage. In 1989, beaver induced flooding was responsible for an estimated annual loss of $22 million to the timber industry. In the pacific northwestern U.S., mountain beaver (*Aplodontia rufa*), pocket gopher and porcupine (*Erethizon dorsatum*) damage Douglas-fir (*Pseudotsuga menziesii*), lodgepole pine (*Pinus contorta*) and ponderosa pine(*Pinus ponderosa*) at all

stages of tree growth. In 1989, the U.S. Department of Agriculture spent $6.2 million to reduce timber damage by mammals on Forest Service land in the pacific northwest (5).

Rodents are implicated in the transmission of diseases such as hantavirus and bubonic plague to man. Damage to buildings and other man-made structures are attributed to rodent nesting, burrowing and feeding activities (6). In 1971 and 1972, Moore et al. (7) surveyed animal damage in the eastern U.S. and found an annual average of 4,203 incidences of rodent bites to humans. Their report of 10.6 rodent bites/100,000 people far exceeds the number of attacks by bears (*Ursidae*), coyotes (*Canis latrans*), or mountain lions (*Felis concolor*) in the U.S.(8). Extrapolation of data to the entire U.S. population indicates that about 26,700 people are bitten in the U.S. annually by rodents (9).

A variety of approaches are utilized in attempts to reduce rodent populations and ultimately rodent damage in the United States. These include exclusion, habitat modification, trapping, shooting and toxicants (10). As shooting, trapping and exclusion are time consuming and practical only in small areas, poisoning alone or in conjunction with habitat manipulation is often the most practical approach for alleviating rodent damage (4).

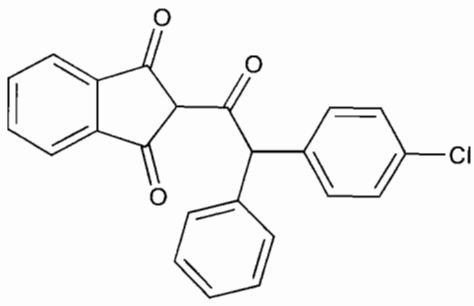
From the early to mid 1900's, millions of hectares of U.S. rangeland were treated with poisoned grains, most frequently strychnine, to control rodent populations. From 1955 to 1965, sodium monofluoroacetate (Compound 1080) was widely used to control rangeland rodent populations. In 1972, U.S. Presidential Executive Order II 11643 banned the use of secondary poisons on federal lands and eliminated most uses of strychnine and sodium monofluoroacetate. In the early 1950's, the anticoagulant Warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin)gained widespread acceptance and was successfully used against many rodents, especially commensal species. Warfarin and other first generation anticoagulants were characterized by moderate toxicity (10-50 mg/kg) and repeated doses increased toxicity. After about 10-25 years of use, resistance in some pest rodent populations has become troublesome. To minimize resistance, there is a need for new rodenticides which do not exhibit cross resistance with Warfarin and preferably have increased toxicity so as not to require multiple dosing (11,12).

U.S. Registration Requirements for Rodenticides

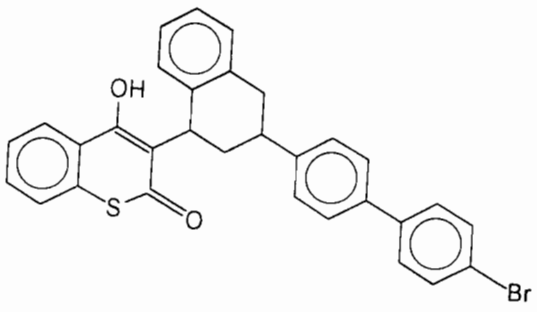
The U.S. Environmental Protection Agency (EPA) requires specific data (40 CFR Part 158 of FIFRA) to support registration for pesticides (including rodenticides) to be used in the U.S. Data requirements are determined by primary (terrestrial or aquatic) and secondary (food or nonfood) uses for each active ingredient and end use product. The basic data requirements for all pesticides include: 1) Product Chemistry Studies to determine the physical and chemical characteristics of the active ingredient and impurities; 2) Wildlife and Aquatic Organism Toxicity Studies to determine toxicity to nontarget organism (laboratory and field studies); 3) Toxicology and Human Health Hazard Studies to assess hazards according to duration and route of exposure; 4) Nontarget Plant Hazard Evaluation to determine pesticide effects on seed germination and plant vigor; 5) Environmental fate studies to determine

potential movement, degradation/ metabolism of pesticides in the environment; and 6) Residue Chemistry studies to determine pesticide residues in plants and animals to determine acceptable residues levels for all food items.

In addition to the significant costs to generate the required data, the U.S. EPA requires a registration fee of $50,000 - $150,000 for each active ingredient plus annual registration maintenance fees ranging from $700 - $1,400. Compared to most insecticides, fungicides and herbicides, rodenticides are minor use compounds. Due to the small volumes of rodenticides used, it is not economically feasible for many manufacturers to pursue the costs of registration, potentially leading to a void of rodenticides to be used by the agricultural community, public health professionals and professional pest control personnel as well as private citizens to control pest rodent populations.(13) To fill this void, scientists at the National Wildlife Research Center (U.S. Department of Agriculture/Animal Plant Health and Inspection Service/Animal Damage Control) are assisting in generating the data required for registration of new rodenticides for animal damage management. Under cooperative funding provided by the California Vertebrate Pest Advisory Council and Liphatech, we are conducting field and laboratory studies to support the registration of the indandione anticoagulant chlorophacinone (2-(α-4-chlorophenyl-α-phenylacetyl)-1,3-indandione) and the coumarin anticoagulant difethialone (1'hydroxy-4-coumarin, 3-[3-{4'-bromo(1,1'biphenyl)-4-yl}-1,2,3,4-tetrahydro-1-napthalenyl}-4-hydroxy-2H-1-benzothiopyran-2-one). The structures of these compounds are illustrated in figure 1. To assess the potential exposure of grazing



CHLOROPHACINONE



DIFETHIALONE

Figure 1. Anticoagulants chlorophacinone and difethialone

livestock to anticoagulant residues following field applications, we were required to develop a residue method for chlorophacinone in rangegrass. To determine the potential for secondary hazards due to predatory wildlife feeding on poisoned species, we developed an analytical method to quantify chlorophacinone residues in poisoned ground squirrels and difethialone residues in poisoned rats (*Rattus norvegicus*). To fill the data gap concerning the toxicity of difethialone to potential secondary consumers of poisoned target species, we

developed methodology to quantify difethialone in difethialone fortified dog food to be used for toxicity testing in the potential secondary consumers magpies (*Pica pica*) and ferrets (*Mustela putorius furo*).

Solid Phase Extraction

Traditionally, liquid-liquid partition chromatography has been the heart of most pesticide methods. Recently, solid phase extraction (SPE) columns have been used to improve sample preparation techniques for isolating pesticides and pharmaceuticals from relatively clean aqueous matrices. SPE technology typically incorporates nonpolar, polar or ion-exchange sorbents onto a silica substrate via a silyl ether linkage to prepare sorbents which are loaded into disposable columns. Most SPE methods utilize fourbasic steps. To improve reproducibility, the columns are conditioned with the solvents to be used for loading and elution. Next, the sample extract containing the analytes and matrix extractants is loaded onto the column. The column is then rinsed with a weak solvent that will elute the matrix extractants and leave the analytes on the column (or elute the analytes and leave matrix extractants on the column). Finally, a slightly stronger solvent is used to elute the analytes and leave additional matrix extractants on the column. Compared to liquid-liquid extraction, solid phase extraction offers time savings, solvent reduction, high selectivity, sample concentration, elimination of emulsions and the potential for automation (14,15). In our laboratory, we have adapted SPE methodology for the quantification of pesticides in very difficult matrices: whole body animals and plants. These matrices require significant sample cleanup to isolate and subsequently quantify rodenticide residues at parts per billion levels.

METHODOLOGY

Chlorophacinone in California Rangegrass

Sample Preparation & Extraction. Frozen California rangegrass was ground (Model RSI 6V, Robot Coupe Inc., Ridgeland, MS) in 100 g aliquots until all pieces were $\leq$ 2 cm in length. Ground grass was stored at $\leq$ -15°C in a sealed stainless steel container. Aliquots of 1.0 - 1.1 g ground grass were weighed into 50 mL screw top glass test tubes. Chloroform (10 mL) was added, the tubes were capped and shaken horizontally on a mechanical mixer for 45 minutes. The tubes were centrifuged at 2500 rpm for 5 min and approximately 7 mL of supernatant was filtered through a 45μm teflon filter into a 10 mL screw top centrifuge tube.

Sample Cleanup & Analyte Recovery. Aminopropyl (NH_2) (0.5 g) SPE columns (International Sorbent Technology, Jones Chromatography, Lakewood, CO) were conditioned with 5 mL chloroform. Then 5 mL of the filtered sample extract was eluted through the SPE column at 1 - 2 mL/min. Colored matrix extractants were washed from the SPE column with 10 mL chloroform followed by 4 ml ethyl acetate and finally 4 mL methanol. Chlorophacinone was recovered from the column by elution with 5 mL 5 mM tetrabutyl ammonium dihydrogen phosphate in methanol. The chlorophacinone containing eluate was reduced to dryness under nitrogen at 50°C, reconstituted in 1.0 mL HPLC (high performance liquid chromatography) mobile phase, filtered through a 0.45 μm teflon filter and transferred to an autosampler vial.

Analyte Quantification. The rangegrass extracts were analyzed by HPLC using a Hewlett Packard (Sunnyvale, CA) 1090 HPLC system equipped with a DAD detector

and Hewlett Packard PC and Chemstation software. A C18/Hypersil Keystone (Bellefonte, PA) 250 mm x 4.6 mm analytical column and guard column were used with a flow rate of 1.0 mL/min 5 mM tetra-butylammonium dihydrogen phosphate in 80:20 methanol:water (pH = 7.5). Column temperature was maintained at 35 °C and injections volumes were 25 μL. Chlorophacinone was quantified by uv absorbance at 285 nm.

Analyses of control plants indicated that there were no matrix interferrants at the retention time of chlorophacinone (6.4 min). Detector response was linear ($r^2 \geq 0.99$) for chlorophacinone solutions containing 0.03 to 10 μg/mL. Ground plant tissue aliquots were fortified with chlorophacinone at 0.1, 1.0 and 10 μg/g. The analyses of seven replicates at each fortification level yielded a mean recovery of 93.6%. The method limit of detection for chlorophacinone in California range grass was approximately 15 ppb.

Chlorophacinone in California Ground Squirrels

Sample Preparation & Extraction. To prepare whole frozen ground squirrels for analyses, carcasses were partially thawed and the pelt, head and extremities removed. The remaining whole body was ground until homogeneous with a variable speed batch sample processor (Model RSI 6V, Robot Coupe Inc., Ridgeland, MS). The tissue homogenate was then frozen with liquid nitrogen and powdered in a nitrogen mill (16). Aliquots of approximately 1 g powdered tissue were combined with 10 g anhydrous sodium sulfate and ground/mixed with a mortar and pestle. This mixture was transferred to a 50 mL centrifuge tube and combined with 3 x 5 mL 1:1:0.005 chloroform:acetone:88% formic acid rinses of the mortar and pestle. The tube was capped, mechanically shaken for 20 minutes and centrifuged at 2500 rpm for 5 minutes. The tissue extraction was repeated two more times with 10 mL extraction solvent. The extracts were combined and evaporated to dryness at 50°C under a gentle stream of nitrogen. The residue was dissolved in 5 mL hexane.

Sample Cleanup & Analyte Recovery. Silica (1 g) SPE columns (International Sorbent Technology, Jones Chromatography, Lakewood, CO) were conditioned with 5 mL hexane. The reconstituted sample extract was eluted through the column at 1 - 2 mL/min. The column was washed with 12 mL of hexane. Hexane eluants were discarded. Chlorophacinone was recovered from the column by elution with 20 mL 15% methanol in ether. The chlorophacinone containing eluate was reduced to dryness under nitrogen at 50°C, reconstituted in 1.0 mL HPLC mobile phase, filtered through a 0.45 μm teflon filter and transferred to an autosampler vial.

Analyte Quantification. The whole body squirrel extracts were analyzed by HPLC using the same instrumentation and conditions used for the analysis of range grass extracts except that the mobile phase was 5 mM tetra-butylammonium dihydrogen phosphate in 68:32 methanol:water and injections volumes were 100 μL.

Analyses of control animals indicated that there were no matrix interferrants at the retention time of chlorophacinone (15.0 min). Detector response was linear ($r^2 \geq 0.99$) for chlorophacinone solutions containing 0.03 to 10 μg/mL. Tissue homogenates were fortified with chlorophacinone at 0.1, 1.0 and 10 μg/g. The analyses of seven replicates at each

fortification level yielded a mean recovery of 84.4%. The method limit of detection for chlorophacinone in ground squirrels was approximately 60 ppb.

Difethialone in Rats

Sample Preparation & Extraction. Animals were processed into homogenates as described for ground squirrels and 2.0 g aliquots were transferred to 50 mL screw top glass tubes. A 5% ascorbic acid solution (100 μL) and 4.5 g sodium sulfate were added to each tube. The tubes were mixed thoroughly on a vortex mixer and 15 mL 1% formic acid in chloroform:acetone (1:1) was added. The tubes were mixed again and centrifuged at 3000 rpm for 5 minutes. The supernatant was decanted into a 50 mL glass tube. The tissue extraction was repeated two more times with 10 mL formic acid/chloroform:acetone. The supernatants were combined and filtered (0.45 μm teflon filter). The extracts were evaporated to dryness under a gentle stream of nitrogen at 50°C and reconstituted in 10 mL hexane by mixing and sonication.

Sample Cleanup & Analyte Recovery. Silica (2 g) SPE columns (International Sorbent Technology, Jones Chromatography, Lakewood, CO) were conditioned with 5 mL of hexane. The entire hexane extract was eluted through the column at 1-2 mL/min. Potential matrix interferants were removed by washing with 10 mL 20% ether in hexane. Difethialone was recovered by elution with approximately 23 mL of 90% ethyl ether in hexane into a 25 mL glass test tube. The tube contents were evaporated to dryness and reconstituted in 5 mL hexane.

NH_2 (1 g) SPE columns (International Sorbent Technology, Jones Chromatography, Lakewood, CO) were conditioned with 5 mL hexane. The hexane reconstituted silica SPE eluate was eluted through the NH_2 column. The column was sequentially washed with 10 mL chloroform:2-propanol (2:1), 5 mL chloroform, 5 mL ethyl acetate and finally 6 mL of 2% acetic acid in ether. Difethialone was recovered by elution with 8 mL of 5% ammonium hydroxide in methanol. The difethialone containing eluate was evaporated to dryness, reconstituted in 2 mL mobile phase, filtered (0.45 μm teflon filter) and transferred to an autosampler vial.

Analyte Quantification. The difethialone containing extracts were analyzed by HPLC using the same instrumentation and conditions as used for the analysis of ground squirrel extracts except that the mobile phase was 5 mM tetrabutylammonium dihydrogen phosphate in 77:23 methanol:water and injections volumes were 200 μL. Column temperature was 40°C. Difethialone was quantified by uv absorbance at 262 nm.

Analyses of control rats indicated that there were no significant matrix interferrants at the retention time of difethialone (20.5 min). Detector response was linear ($r^2 \geq 0.99$) for difethialone solutions containing 0.100 to 30 μg/mL. Ground tissue aliquots were fortified with difethialone at 0.2, 1.0 and 20 μg/g. The analyses of seven replicates at each fortification level yielded a mean recovery of 76.2%. The method limit of detection for difethialone in rats was approximately 50 ppb.

Difethialone in Dog Food

The procedure for the analysis of difethialone in canned dog food was

identical to the rat method except that the quantity of sodium sulfate used was increased to 10 g, the ethyl acetate wash was omitted and the volume of the 2% acetic acid in ether wash was increased to 8 mL. Detector response was linear ($r^2 \geq 0.99$) for difethialone solutions containing 0.050 to 220 μg/mL. Dog food was fortified at 0.1, 10, and 100 μg/g. The analyses of seven replicates at each fortification level yielded a mean recovery of 83.9%. The method limit of detection for diphacinone in dog food was approximately 85 ppb.

DISCUSSION

As indicated by the chromatograms in figure 2, solid phase extraction coupled with ion-pair reversed phase HPLC provided excellent cleanup and sensitivity for the analysis of both chlorophacinone and difethialone in very difficult animal and plant matrices. The total volume of organic solvents required and subsequently disposed as hazardous waste was 39 mL for the analysis of chlorophacinone residues in range grass, 78 mL for the analysis of chlorophacinone in ground squirrels (and livers), and 129 mL for the analysis of difethialone residues in rats. This is a significant improvement compared to traditional methods which utilize solvent extraction followed by gel permeation chromatography (GPC) to separate the rodenticides from lipid coextractants. For example, this solvent extraction/GPC approach was used by Hunter (17,18) to quantify chlorophacinone in rodent livers. Sample preparation required 428 mL of organic solvent per sample. By substituting SPE for GPC, we achieved a 550% reduction in solvent usage. Recoveries from chlorophacinone fortified tissues were comparable for the two methods.

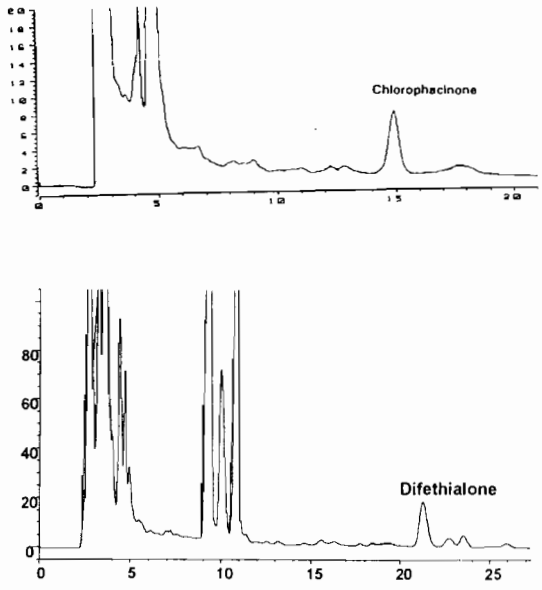


Figure 2. Chromatograms from the HPLC analysis of chlorophacinone fortified squirrel extract (top) and difethialone fortified rat extract (bottom)

The addition of acid to the sample matrix during extraction improved the recoveries of anticoagulants from animal tissues. Formic acid was used to enhance chlorophacinone and difethialone recoveries in ground rodent tissues. The addition of ascorbic acid also improved the recovery of difethialone from rats. It is believed that the addition of these acids improve recoveries by preventing significant complexation of the analytes with oxidized heme as the acids maintain heme in its reduced state (18). Acidified extracts may also help to minimize rodenticide binding to other proteins as well (19). Acidification of the extraction solvent did not improve the extraction efficiency of chlorophacinone from range grass, presumably due to the absence of

heme and lower levels of protein content of the grass.

The addition of sodium sulfate was essential for adequate analyte recoveries from animal tissues. Sodium sulfate binds the water in these tissues (20) which would otherwise interfere with the solvent extraction of the analytes. Sodium sulfate was not added to the grass matrix as the water content was negligible. More sodium sulfate was required for consistent recoveries from dog food as compared to the whole body rat tissue homogenates as the dog food had a higher moisture content. A NH_2 SPE column worked best for the cleanup and recovery of chlorophacinone from range grass while a silica SPE column worked better for ground squirrel samples. The NH_2 SPE afforded superior retention of the colored plant coextractants while silica appeared to better retain the coextracted animal lipids. While the primary retention mechanisms for the isopropyl amine sorbents are polar and ion-exchange (pKa = 9.8), the isopropyl chain provides for a weak non-polar retention mechanism. This non-polar character likely provides for the retention of plant pigments as chlorophylls and carotenoids posses long non-polar chains which are well suited to non-polar interactions. Silica's retention mechanism is exclusively polar interactions. On a weight basis, silica sorbents provide more active sites than other SPE sorbents, making silica well suited for retention of large quantities of lipids which are coextracted from whole body homogenates. To permit the retention of lipids, the loading solvent was the non-polar solvent hexane.

Both silica and NH_2 SPE technologies needed to be combined to provide sufficient cleanup and recovery of difethialone from rat tissue and dog food. Likely this is due to the fact that difethialone is less polar than chlorophacinone; it was more difficult to isolate difethialone from the relatively non-polar lipids in the animal tissue homogenates. However, judicious use of wash solvents for the NH_2 column permitted the removal of classes of lipids which have a significant potential negative impact on chromatography. During the loading of the difethialone extract in hexane, difethialone and all lipids were retained on the NH_2 column. The chloroform:2-propanol wash removed the lipids which were retained primarily by polar interaction, cholesterol esters, triglycerides, cholesterol, diglycerides and monoglycerides. The acetic acid:ether wash increased the polarity of the mobile phase and/or protonated the free fatty acids. This minimized retention by anion-exchange, subsequently washing the free fatty acids from the column (21). The ammonium hydroxide:methanol wash significantly increased the polarity of the mobile phase, eluting the difethialone along with small amounts of phospholipids (22). Also, a longer HPLC retention time was needed to resolve difethialone from co-extractants than was needed for chlorophacinone, resulting in a higher instrument limit of detection for difethialone. To achieve a comparable method limit of detection for both compounds, a larger sample needed to be analyzed for the difethialone. This too contributed to the need for a more rigorous cleanup for the analysis of difethialone in animal tissues.

CONCLUSION

Solid phase extraction offers a variety of retention mechanisms useful for the selective cleanup of both coumarin and indandione anticoagulant rodenticides from

complex biological matrices. The addition of sodium sulfate and ascorbic/formic acids minimized extraction problems associated with matrix water and proteins, respectively. Capitalizing on the polar and ionic nature of various lipid classes and SPE solid supports permitted the development of cleanup procedures leading to the selective concentration of the rodenticide residues. These methods are applicable to a wide variety of pesticide registration studies as well as forensic studies involving both primary and secondary poisonings.

REFERENCES

1. Fagerstone, K.A. and Connolly, G. In: Brewer, L.W. and Fagerstone, K.A. (Editors) Avian Radiotelemetry, SETAC Special Publication Series, Louis Publishers, CRC Inc., Boca Raton, FL. (In Press)
2. National Agricultural Statistics Service. 1990. Highlight of the Animal Damage Control Survey. U.S. Department of Agriculture. 2pp
3. Elias, D.J. and Fall, M.W. 1988. In:Prakash, I. (Editor) Rodent Pest Management, pages 13-24, CRC Press, Boca Raton, FL.
4. Fagerstone, K.A. and Ramey, C.A. 1996. In: Krausman, P.R. (Editor) Rangeland Wildlife, pages 83-132, The Society of Range Management, Denver, CO.
5. Borrecco, J.E. and Black, H.C. 1990 *Proc. Vertebr. Pest Conf.* 14, 192-198.
6. Marsh, R.E. In:Prakash, I. (Editor) Rodent Pest Management, pages 1-12, CRC Press, Boca Raton, FL.
7. Moore, R.M., Zehmer,R.B., Moulthroup, J.I. and Parker, R.L. 1977. *Arch. Environ. Health* 32, 267-270
8. Conover, M.R., Pitt, W.C., Kessler, K.K., DuBow, T.J. and Sanborn, W.A. 1995. *Wildlife Society Bulletin* 23 (3), 407-414.
9. Herrero, S. 1985. Bear Attacks. Nick Lyons, N.Y., N.Y., 281pp.
10. Prevention and Control of Wildlife Damage, Vol I. 1994. Hygnstron, S.E., Timm, R.M. and Larson, G.E. (Editors), University of Nebraska, Lincoln, NE. B1-187)
11. Lund, M. 1988 In:Prakash, I. (Editor) Rodent Pest Management, pages 331-340, CRC Press, Boca Raton, FL.
12. Lund, M. 1988 In:Prakash, I. (Editor) Rodent Pest Management, pages 341-351,CRC Press, Boca Raton, FL.
13. Fagerstone, K.A., Bullard, R.W. and Ramey, C.A. 1990. In: Davis, L.R. and Marsh, R.E (Editors) Proc. 14th Vertebr. Conf. U.C. Davis, pages 8-11, Davis, CA.
14. Hooper, M.L. 1992. In: Cairns, T. and Sherma, J. (Editors) Emerging Strategies for Pesticide Analysis, pages 39-50.
15. Stewart, J.T., Reeves, T.S. and Honigberg, I.L. 1984. *Analytical Letters* 17(B16) 1811-1826.
16. Sterner,R.T and Mauldin, R.E. 1995. *Archives of Environmental Contamination and Toxicology* 28, 519-523.
17. Hunter, K. 1985. *J. Chromatogr.* 321, 255-272.
18. Hunter, K. and Sharp, E.A. 1988. *J. Chromatogr.* 437, 301-305.
19. Bullard, R.W., Holguin, G. and Peterson, J.E. 1975. *J. Agric. Food Chem.* 23,72-75.
20. Hoogenboom J.L. and Rammell, C.G. 1983. *Bull. Environ. Contam. Toxicol.* 31, 239-243.
21. Kaluzny, M.A., Duncan, LA., Merritt, M.V. and D.W. Epps. 1985. *J. Lipid*

Res. 26, 135-140.

22. Lehninger, A.L. 1978. In: Biochemistry, The Molecular Basis of Cell Structure and Function 2nd edition, pages 279-308, Worth Publishers, N.Y., N.Y.